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Evaluation of *Alternaria* and Its Mycotoxins During Ensiling of Sunflower Seeds

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ABSTRACT Sunflower (*Heliantus annuus*) is an important crop in the economy of Argentina due to its high production and demand from domestic and export markets. The aim of this study was to evaluate the presence of *Alternaria* species and its mycotoxins in ensiled sunflower seeds. The sampling was carried out in three periods: at the beginning of ensiling, on the second month and finally on the fourth month. The mycological analysis was made with disinfected seeds, cultured on Dichloran-Rose Bengal-Chloramphenicol (DRBC) and Dichloran-Chloramphenicol-Malt Extract-Agar (DCMA). The toxins were analyzed by thin layer chromatography (TLC). *A. alternata* was the main fungal species isolated. The incidence of *Alternaria* species and the levels of alternariol and tenuazonic acid decreased as the time of ensiling increased. Alternariol monomethyl ether was detected in two samples from the second and third sampling periods. *Nat. Toxins 5:20–23, 1997.*

Key Words: oil seeds ensiling; mycobiota; *Alternaria alternata;* alternariol (AOH); alternariol monomethyl ether (AME); tenuazonic acid (TA)

INTRODUCTION

In Argentina sunflower seeds fill a important place in the national and regional economy due to the great demand for seeds and by-products (oil and meal) in domestic and export markets [Bolsa de Cereales, 1994]. This substrate is susceptible to *Alternaria* contamination [Logrieco et al., 1988; Dalcero et al., 1989]. The genus *Alternaria* is ubiquitous and includes both plant pathogenic and saprophytic species that may damage crops in the field or cause post-harvest decay. Moreover, several species have also been widely observed as spoilage agents of cereals, fruits and vegetables in storage or during refrigerated transport. Certain species are capable of producing toxic metabolites in infected plant and in agricultural commodities, which can contaminate food and feed and elicit adverse effects in animals [Bottalico and Logrieco, 1992; Visconti and Sibila, 1994]. Among the mycotoxins synthesized by *Alternaria*, seven are naturally occurring. Three of them, alternariol (AOH), alternariol monomethyl ether (AME) and tenuazonic acid (TA), have been detected in freshly harvested sunflower seeds, with contamination frequencies of 85%, 45% and 66%, respectively [Chulze et al., 1995]. The distribution of these mycotoxins was also evaluated in sunflower by-products. The toxins were found in meal, with 88–92% of AOH, 44–48% of AME and 64–47% of TA in the samples tested [Montemurro and Visconti, 1992; Torres et al., 1993; Chulze et al., 1995].

Although the optimum conditions of water activity (a_w) and temperature for the production of these mycotoxins before storage are known [Torres et al., 1992; Etcheverry et al., 1994], there are no data on their stability in stored seeds. This information only exists in ensiling sunflower seeds in relation to the contamination with *Aspergillus flavus* and aflatoxins, indicating that a_w, temperature and microbial activity can affect the concentration of these toxins [Etcheverry et al., 1989, 1996].

The aim of the present study was to determine the occurrence of *Alternaria* species in stored sunflower seeds and to evaluate their growth and production of mycotoxins during this time.

MATERIALS AND METHODS

Sampling

Sunflower seeds

The samples (1994 crop from Córdoba Province) were collected at the moment when the silo was filled up and during the second and fourth months of storage. Humidity and temperature were controlled. During the first, second and third samplings, the moisture contents were of 12%, 9%

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TABLE I. Occurrence of AOH, AME and TA in Sunflower Seeds During Storage

	Sampling								
	First			Second			Third		
Subsample	AOH	AME	TA	AOH	AME	TA	AOH	AME	TA
1	980	ND	31600	800	600	15800	1600	800	6240
2	980	ND	31600	600	ND	15800	1066	ND	6240
3	980	ND	23400	600	ND	15800	800	ND	5820
4	680	ND	23400	600	ND	9500	800	ND	4680
5	680	ND	23400	600	ND	9500	ND	ND	4680
6	680	ND	23400	600	ND	7800	ND	ND	3120
7	680	ND	15600	400	ND	7800	ND	ND	3120
8	680	ND	15600	400	ND	3660	ND	ND	3120
9	680	ND	15600	400	ND	3660	ND	ND	3120
10	680	ND	7800	400	ND	3660	ND	ND	ND
11	680	ND	7800	160	ND	3660	ND	ND	ND
12	680	ND	7800	100	ND	3660	ND	ND	ND
13	680	ND	7800	100	ND	3600	ND	ND	ND
14	680	ND	7800	100	ND	ND	ND	ND	ND
15	680	ND	7800	100	ND	ND	ND	ND	ND
16	680	ND	3900	100	ND	ND	ND	ND	ND
17	680	ND	ND	60	ND	ND	ND	ND	ND
18	250	ND	ND	ND	ND	ND	ND	ND	ND
19	250	ND	ND	ND	ND	ND	ND	ND	ND
20	250	ND	ND	ND	ND	ND	ND	ND	ND
Means	661	—	16000	360	—	8000	1070	—	4500

Values are means of duplicates and are expressed as μg/kg.
ND, not detected. Detection limits (μg/kg): AOH, 50; AME, 30; TA, 100. AOH and TA values were significantly different in the three samplings (Kruskal-Wallis test: $P = 0.0003$ and $P = 0.0006$ respectively).

and 7% and the temperatures were 30°C, 20°C and 15°C respectively. Twenty representative samples of 10 kg each were taken, homogenized and ground for each time period. A uniform 1 kg subsample was obtained and was ground again to pass through a 20 mesh sieve, and samples of 50 g were removed for toxin analysis. All samples were stored at 4°C until the analysis was carried out. Five of the 20 above samples were taken to analyze the mycoflora of the sunflower seeds in the silo.

Sunflower meal and oil

The samples were taken when the seeds were processed. The processing of 80,000 tons of sunflower seeds took 6 days. To obtain the by-products the sunflower seeds are put under the following treatment. Once the seeds have been shelled they receive a 100°C heat treatment. After they are pressed, the speller is obtained; then, by adding hexane, meal and oil are obtained. After this process, 20 representative samples of 10 kg of meal and 1 L of oil were taken every 12 hours.

Isolation and Identification of *Alternaria* Species From Sunflower Seeds

Each sample (50 seeds) was superficially disinfected (2% sodium hypochlorite, 1 minute) and placed in two culture media: Dichloran-Rose Bengal-Chloramphenicol (DRBC), recommended for fungal general count in foods [Pitt and Hocking, 1985], and Dichloran-Chloramphenicol-Malt Extract-Agar (DCMA), recommended for the isolation of *Alternaria* spp. [Andrews, 1992]. Petri dishes were incubated at 25°C during 5–7 days.

The *Alternaria* isolates were subcultured on Potato-Dextrose-Agar (PDA) and the cultures were taxonomically classified following keys of Ellis [1976] and Simmons [1990].

AOH, AME and TA Analyses

Sunflower seeds and meal

The mycotoxins were determined using the method by Visconti et al. [1986]: 25 g of ground sunflower seeds or meal were homogenized with 75 ml of methanol in an oscillating shaker. The homogenate was filtered and 30 ml of the filtrate was stirred for 1 minute with 60 ml of 20% aqueous ammonium sulfate and again filtered. The fat from the filtrate was removed by extraction with 30 ml of hexane. The hexane layer was discarded and the aqueous phase was extracted twice with 5 ml of chloroform. The remaining aqueous solution after chloroform extraction was acidified to pH 2 with concentrated hydrochloric acid and extracted

twice with 50 ml methylene chloride. TA was extracted from the combined organic phases with 30 ml of 5% aqueous sodium bicarbonate, then reconverted to its acidic form by adjusting to pH 2 with 1 N hydrochloric acid, and extracted twice with 30 ml methylene chloride. The methylene chloride extracts were combined, washed with 25 ml of water, and evaporated to dryness. The residue was dissolved in 200 µl of methanol and analyzed by thin layer chromatography (TLC) using the same conditions described above.

Sunflower oil

The mycotoxins were extracted with hexane (60 ml), water (40 ml), methanol (60 ml) and concentrated hydrochloric acid (1 ml) in a separatory funnel. The oil layer was discarded and the aqueous phase was re-extracted with hexane (60 ml), which was discarded. The aqueous phase was re-extracted twice with 50 ml chloroform, and the combined extracts were evaporated to dryness. The residue was dissolved in 200 µl of methanol and analyzed by TLC using the same conditions described above.

Statistical Analysis

The mycotoxin data were analyzed applying nonparametric tests: Kruskal-Wallis and Miller multiple comparisons [Hamilton, 1993; Hollander and Wolfe, 1972].

RESULTS AND DISCUSSION

When the incidence of *Alternaria* spp. was studied in sunflower seeds, a gradual decrease in the isolation frequency during the period of silage was detected. The initial contamination rate was 60% and it decreased to 49% after 2 months and 36% after 4 months of storage.

Together with *Alternaria*, other Demateaceous species belonging to *Drechslera, Cladosporium* and *Ulocladium* were isolated. *Aspergillus, Penicillium, Mucor* and yeasts were found with low frequency, from DRBC only. Among *Alternaria* isolates, 90% were identified as *A. alternata.* This study showed that the *Alternaria* mycotoxins in ensiling seeds during the first period had a high natural contamination of 100% for AOH and 80% for TA, with average toxin levels of 661 µg/kg and 16,000 µg/kg respectively. During the second and third samplings there was a gradual decrease in both toxins, found it for 85% (average toxin levels 360 mg/kg) and 20% (average toxin levels 1,070 mg/kg) for AOH respectively and found it for TA 65% (average toxin levels 8,000 mg/kg) and 45% (average toxin levels 4,500 mg/kg) respectively. AME was detected only in two samples corresponding to the second and third samplings, with toxin levels of 600 µg/kg and 800 µg/kg respectively (Table I).

Although Chulze et al. [1995] demonstrated the presence of AOH in sunflower meal, in our hands the toxins AOH, AME and TA were not detected in meal and oil samples obtained from the ensiled sunflower seeds.

Although a large number of studies have been done on AOH, AME, TA and other *Alternaria* metabolites [King and Schade, 1984; Torres et al., 1993; Chulze et al., 1995], this is one of the first reports on the stability of the toxins in this oil seed. Only one study was reported on the incidence of AOH, AME, ATX-I and TA in wheat flour stored during 28 days at 20°C; a reduction of 30–40% of the metabolites was obtained [Stinson and Heisler, 1981]. Another mycotoxin such as zearalenone has been demonstrated to disappear during grain storage [Scott, 1991]; this toxin, as *Alternaria* toxins, is produced before storage.

Our results suggest that the toxins of *Alternaria*, AOH and TA, could break down during ensiling of sunflower seeds, and that a good storage would lower the levels of these *Alternaria* toxins, decreasing the risk of obtaining contaminated by-products. The treatment used to obtain sunflower by-products could reduce AOH, AME and TA concentrations over those in the raw material.

REFERENCES

Andrews S (1992): Differentiation of *Alternaria* species isolated from cereals on Dichloran Malt Extract Agar. In Samson RA, Hocking AD, Pitt JI, King AD (eds): ''Modern Methods in Food Mycology.'' Amsterdam: Elsevier, pp 351–355.

Bolsa de cereales de Buenos Aires. Número estadístico (1994): Buenos Aires, República Argentina.

Bottalico A, Logrieco A (1992): *Alternaria* plant diseases in Mediterranean countries and associated mycotoxins. In Chelkowski J, Visconti A (eds): ''*Alternaria* Biology, Plant Diseases and Metabolites.'' Amsterdam: Elsevier, pp 209–232.

Chulze S, Torres A, Dalcero A, Etcheverry M, Ramirez M, Farnochi C (1995): *Alternaria* mycotoxins in sunflower seeds: Incidence and distribution of the toxins in oil and meal. J Food Protect 58:1133–1135.

Dalcero A, Chulze S, Etcheverry M, Farnochi C, Varsavsky E (1989): Aflatoxins in sunflower seeds: Influence of *Alternaria alternata* on aflatoxin production by *Aspergillus parasiticus.* Mycopathologia 108: 31–35.

Ellis MB (1976): ''More Demateaceous Hyphomycetes.'' Surrey, England: Mycological Institute.

Etcheverry M, Dalcero A, Chulze S, Apro N, Fusero S, Farnochi M (1989): Studies on damage to sunflower seeds: Water activity, germination, acidity index and aflatoxin B_1 presence. Int J Food Microbiol 8:363–365.

Etcheverry M, Chulze S, Dalcero A, Varsavsky E, Magnoli K (1994): Effect of water activity and temperature on tenuazonic acid production by *Alternaria alternata* on sunflower seeds. Mycopathologia 126:179–182.

Etcheverry M, Chulze S, Dalcero A (1996): Influencia de la concentración del inóculo sobre el crecimiento y la acumulación de aflatoxinas. Revista Iberoamericana de Micología 13:18–23.

Hamilton LC (1993): ''Statistics With Stata 3.'' Belmont, Calif.: Duxbury, pp 59–72.

Hollander M, Wolfe D (1972): ''Nonparametric Statistical Methods.'' New York: John Wiley, pp 115–124.

King AD, Schade JE (1984): *Alternaria* toxins and their importance in food. J Food Protect 47:886–901.

Logrieco A, Bottalico A, Visconti A, Vurro M (1988): Natural occurrence of *Alternaria* mycotoxins in some plant products. Microbiol Alim Nutr. 6:13–17.

Montemurro N, Visconti A (1992): *Alternaria* metabolites, chemical and biological data. In Chelkowski J, Visconti A (eds): ''*Alternaria*, Biology, Plant Diseases and Metabolites.'' Amsterdam: Elsevier, pp 451–560.

Pitt JI, Hocking AD (1985): ''Fungi and Food Spoilage.'' London: Academic.

Scott PM (1991): Possibilities of reduction or elimination of mycotoxins present in cereal grain. In Chelcowski J (ed): ''Cereal Grain: Mycotoxins, Fungi and Quality in Drying and Storage.'' Amsterdam: Elsevier, pp 529–572.

Simmons E (1990): *Alternaria* themes and variations. Mycotaxon 37:79–119.

Stinson EE, Heisler EG (1981): Stability of *Alternaria* mycotoxins in fruit juices and whole wheat flour during storage and processing. Abstr. 182nd Am. Chem. Soc. Nat. Mtg., New York, pp 231–238.

Torres A, Chulze S, Varsavsky E, Dalcero A, Etcheverry M, Farnochi C (1992): Influencia de la temperatura y actividad acuosa en la producción de micotoxinas por *Alternaria alternata* (Hyphomycetales) en girasol. Boletín de la Asociación Argentina de Botánica 28:175–181.

Torres A, Chulze S, Varsavsky E, Rodriguez M (1993): *Alternaria* metabolites in sunflower seeds. Incidence and effect of pesticides in their production. Mycopathologia 121:17–20.

Visconti A, Sibila A (1994): *Alternaria* toxins. In Miller JD, Trenholm HL (eds): ''Mycotoxins in Grain: Compounds Other Than Aflatoxins.'' St. Paul, MN: Eagan, pp 315–335.

Visconti A, Logrieco A, Bottalico A (1986): Natural occurrence of *Alternaria* mycotoxins in olives—their production and possible transfer into the oil. Food Addit Contam 3:323–330.